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Houston, Texas 77030

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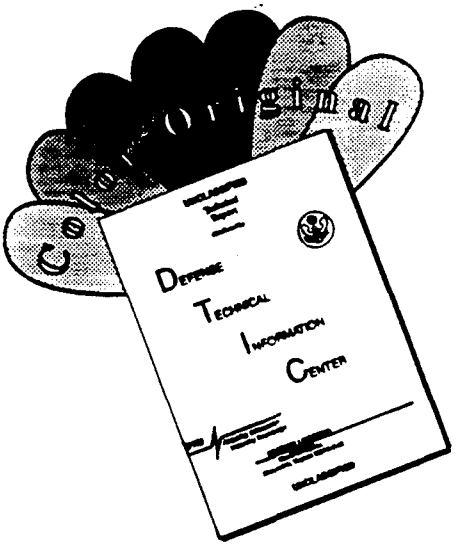

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13. ABSTRACT *(Maximum 200 words)*

This proposal is based upon the hypothesis that the protective effects of an early pregnancy and lactation on the incidence of breast cancer result from estrogen (E) and progesterone (P)-induced differentiation and the resultant loss of cells susceptible to carcinogenesis. These effects of E and P are mediated by the induction of specific "local mediators", i.e., growth factors that act via autocrine and paracrine mechanisms to influence terminal duct (TD) and end bud growth (TEB) and differentiation. These rapidly proliferating cells are the most susceptible to neoplastic transformation. No molecular markers are available to identify and follow the fate of these susceptible cells, yet this information is required to develop effective diagnostic tools and preventive therapies for breast cancer. Thus, the initial objective of this grant is to identify molecular markers for TEB and TD cells in order to follow their fate during mammary development and carcinogenesis. During the first year of this proposal we have validated the feasibility of manually dissecting end bud, mid-gland and stromal subfractions from the mammary glands of 45-50 day old Wistar Furth rats, and the isolation of intact RNA and nuclear matrix proteins from these fractions. We have performed confocal microscopic and differential display PCR and 2D-PAGE analyses and identified differences in gene expression in these fractions. DNA sequencing of unique DD-PCR products has identified a novel member of the rho-GAP family designated p190-B that may be selectively expressed in TEBs.

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Differential Display PCR, Confocal Microscopy, Nuclear Matrix Proteins, Terminal Duct and Terminal End Buds, Breast Cancer

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INTRODUCTION

A woman's reproductive history is one of the principal determinants of her susceptibility to breast cancer. An early full-term pregnancy is protective and the length of time between menarche and the first full term-pregnancy appears to be critical for the initiation of breast cancer. This proposal is based upon the hypothesis that the protective effects of an early pregnancy and lactation result from estrogen (E) and progesterone (P)-induced differentiation and the resultant loss of cells susceptible to carcinogenesis. These effects of E and P are mediated by the induction of specific "local mediators", i.e., growth factors that act via autocrine and paracrine mechanisms to influence terminal duct (TD) and end bud growth (TEB) and differentiation. These rapidly proliferating cells are the most susceptible to neoplastic transformation. No molecular markers are available to identify and follow the fate of these susceptible cells, yet this information is required to develop effective diagnostic tools and preventive therapies for breast cancer. Thus, the initial objective of this grant is to identify molecular markers for TEB and TD cells in order to follow their fate during mammary development and carcinogenesis. To do so, genes expressed specifically in the TEBs of the nulliparous rat mammary gland will be isolated, cloned and characterized. These genes will then serve as molecular markers in TEB cell fate studies. Candidates for TEB molecular markers may include cell cycle factors, proteins which interact with the extracellular matrix, cytoskeletal elements and growth factor receptors.

The following specific tasks were proposed for the first twelve months of this proposal:

Task 1. Identification of molecular markers for TEB and TD cells.

a. Manual dissection of TEBs/TDs, stromal and ductal tree mammary subfractions and their preliminary characterization by confocal microscopy.

b. Isolation of RNA and nuclear matrix proteins.

c. Initial DD-PCR and 2 D PAGE comparative analysis of fractions.

The task listed below was to be initiated six months following the start of this grant, but was not to be completed for 30 months:

Task 2. Characterization of molecular markers for TEB and TD cells.

a. Cloning and sequencing of unique DD-PCR products.

BODY

Task 1a. Manual dissection of TEBs/TDs, stromal and ductal tree mammary subfractions and their preliminary characterization by confocal microscopy.

During this period we have focused our research on developing and improving the methodology needed to dissect specific TEB and TD fractions from whole mammary glands, separate the ductal epithelium from stromal and fat cells, and prepare samples of the nuclear matrix for 2-D PAGE analysis.

1. Mammary gland dissection

To identify molecular markers specific to the TEB, tissue from the fourth abdominal gland of nulliparous 45-50 day old Wistar-Furth rats was used. This is a time point in which TEBs are present in the mammary gland and are highly susceptible to carcinogens. Mammary glands were

visualized by injecting 5% trypan blue into the ducts from the nipple of intact glands. This technique enabled us to visualized the entire gland as shown in Figure 1A-B. From these preparations it was possible to visualize and dissect a regions rich in TEBs, surrounding stroma, and a mid-gland (MG) region. For these experiments, a total of 20 animals were sacrificed and their mammary glands removed and stored in CSK buffer (see below) at -80°C for nuclear matrix preparations. As discussed below, we found that eight glands from four animals were sufficient to run 3-4 2D-PAGE gels for each of the two fractions.

After manual dissection, epithelia and stromal cells were separated by treatment with collagenase. Specifically, the dissected glands were minced with scissors and suspended into DMEM medium containing 0.5% collagenase. After incubation for one hr at 37°C the tissue was passed through a 250μ nylon mesh. The residual mass of tissue was treated with fresh enzyme and further digested for an additional two hrs under the same conditions. Stromal cells were collected from the mesh filtered fraction by centrifugation at 2000 rpm for 4 min. Examples of dissected and digested TEBs are is shown in Figure 1C-D. After treatment, most stromal cells were removed, although some fat cells remained attached to the dissected TEBs. For comparison, 2-D PAGE was carried out on whole glands as well as the TEB fraction and MG fraction as discussed below and shown in Fig. 8-10.

2. Preliminary characterization of rat mammary glands by confocal microscopy.

This phase of Task 1 has been very productive, enabling us, for the first time, to obtain dramatic images of whole mammary glands from the laser scanning confocal microscope as shown in Fig. 2-5. Most of our effort has been aimed at improving the methods for dissecting the mammary glands, fixation and staining using antibody immunofluorescence to identify cytoskeletal proteins and BrdU/anti-BrdU to identify cells in the mammary gland that are in the proliferative phase of the cell cycle.

a) Methods

Our best results have been obtained by removing whole mammary glands from 50 day old rats. In some cases, the whole gland was processed, but results were obtained when specific zones (Figure 1A-B) such as TEBs and mid-gland regions were dissected away from the gland and diced into small segments with a sharp scalpel and permeabilizd in 0.2% Triton X-100 in PBS for 4 min. After washing, the tissues are fixed with 2.5% formaldehyde for 30 min at room temperature; and after washing 3X with PBS, samples are incubated with primary antibodies at various concentrations depending on individual samples.

For DNA staining, rats were injected with BrdU ip (50-100μg/kg) and sacrificed after two hrs. The animals were sacrificed and the glands removed and processed as above. DNA was denatured with 3N Hcl prior to treatment with anti-BrdU FITC mixed with anti-keratin. After staining with the Rhodamine-labeled second antibody, the preparation was examined with a Molecular Dynamics MultiProbe 2001 Inverted Confocal Laser Scanning Microscope.

b) Three-dimensional reconstruction of mammary glands

Figure 2 shows a 3-D projection of a data set collected from 89 optical sections, with each section scanned at a thickness of 1.8μ. This preparation was stained for actin with FITC-labeled phalloidin. Note the details of the alveolar buds and ducts; various blood vessels are shown in the background. Bundles of actin fibers extend along the outer ductal epithelium. These types of projections can be scanned along the z-axis for many microns in order to analyze the DNA as shown in the following figures.

c) DNA synthesis in cells of the TEBs

Fig. 3 shows four consecutive optical sections cut 1.1μ thick through three TEBs. This preparation was labeled with anti-BrdU (FITC) as described above in Methods and counter-stained with anti-keratin 14 (Texas Red). The sample is a 3 hr pulse label with BrdU. The BrdU-labeled nuclei are pseudo-colored green and the keratins are seen in red. It is possible to determine the DNA labeling index (LI), and cell cycle analysis in these types of preparations. Obviously, such regions are very active in DNA synthesis as compared to adjacent areas of the duct and alveolar buds as shown in Fig. 4.

d) DeltaVision deconvolution microscope

In addition to the CLSM, we experimented with a novel fluorescent microscope that produces 3-D images by a computerized process known as deconvolution imaging. The DeltaVision Full Spectrum Optical Sectioning Microscope System developed by Applied Precision, Inc. (Mercer Island, WA) was demonstrated in our laboratory to enable us to compare images of whole mammary glands obtained by both types of microscopy. We were informed that the DeltaVision's image acquisition system would yield higher resolution images at substantially lower illumination intensities, perhaps producing less specimen damage and quenching of the fluorochrome. The deconvolution microscope has the advantage of enabling one to produce 3-D images of mammary glands including the imaging of DNA in the UV spectrum, a process not possible with our Molecular Dynamics CLSM instrument. Figure 5 shows a 3-D reconstruction of TEB triple-stained for keratin 14 (green), actin-phalloidin (red) and DAPI stain for DNA (blue). The images obtained thus far are promising and we will continue to compare results with those obtained with the CLSM. Please note that the images shown in Fig. 5 are from a mouse mammary gland that was used for the preliminary observations.

Task 1b. Isolation of RNA and nuclear matrix proteins

1. The mammary glands from 8-10 rats were visualized for dissection by injection of trypan blue into the primary lactiferous duct. The TEBs, mid gland and fat pad or stroma were manually dissected into separate fractions for RNA isolation as illustrated in Fig.1. Tissue fractions were placed into guanidinium thiocyanate and then onto a cesium chloride cushion for isolation of total RNA. The integrity and purity of the RNA samples were determined by agarose gel electrophoresis.

2. Nuclear matrix proteins were isolated following essentially the same procedures worked out in our laboratory for other cells and tissues, the isolated mammary gland fractions were minced into small pieces in ice-cold cytoskeleton (CSK) buffer (10 mM PIPES, pH 6.8, 100mM NaCl, 300 mM sucrose, 3mM $MgCl_2$, 1mM EGTA, 4mM vanadyl riboside complex, and 1.2 mM PMSF) and homogenized with a pestle on ice in CSK buffer. The nuclei were pelleted by centrifugation at 600 x g for 3 min and subsequently extracted with 0.5% Triton X-100 in the same buffer for 3 min at room temperature. The pellet was then exposed to DNAse I (100 μg/ml) in CSK buffer for 40 min. After incubation with 0.25M ammonium sulfate in the same buffer for 5 min, the samples were washed three times and the final pellet stored at -80°C for future use.

Task 1c. Initial DD-PCR and 2D-PAGE comparative analysis of fractions.

1. DD-PCR

Differential Display PCR (DD-PCR) allows for the identification of differentially expressed mRNA species by the use of primer sets consisting of an 11bp polyT primer plus G, A or C

(T11G, T11A, T11C) and an arbitrary anchored 13mer primer (AP1-8). (See Fig. 6). Total RNA (0.2μg) was used in each T11M primed reverse transcription reaction. Two microliters of the RT reaction was then amplified by PCR using T11M and an anchored primer. The DD-PCR reactions were electrophoresed on 8% acrylamide gels and specific bands were identified by autoradiography.

DD-PCR has been carried out on the three tissue mRNA samples (TEB[E], mid gland[M] and stroma[S]) with 16 different primer sets (AP1 + T11GAC, AP2 +T11GAC, AP3 +T11GAC, AP4 +T11GAC, AP7 +T11GAC and AP8 +T11C). Twenty-nine clones were identified from single sets of RNA samples (e.g. see Fig. 6). Additionally, three other TEB specific clones were isolated from DD-PCR reactions of multiple RNA preparations electrophoresed in tandem, such that the reproducibility of band patterns could be assessed to decrease the potential of false positives (Fig. 7).

2. 2-D gel electrophoresis

Initially, conditions for complete solubilization of nuclear matrix samples in either SDS or urea were carried out. Our best results were obtained by dissolving the nuclear matrix preparation in 2-D sample buffer containing 9 M urea, 4% NP-40, 2%-ME, pH 3-10 ampholytes with protease inhibitors. The first dimensional isoelectric focusing was carried out at 18,000 v hrs after 1.5 hrs of prefocusing. The second dimensional SDS PAGE was run for 5 hrs at a constant temperature of ~12°C. Approximately 150μg of nuclear matrix proteins was loaded for each gel. Gels were stained with high resolution color-based silver stain and processed in the 2-D gel electrophoresis core laboratory in the Department of Cell Biology.

As shown in Fig. 8-9, we have had initial success in producing silver-stained 2-D gels of nuclear matrix proteins from dissected mammary gland from 45-50 day old rats according to our proposed Task 1a. Although preliminary, the methods have been worked out, and the results clearly show significant differences in the protein patterns from tissues taken at different regions of the mammary gland including whole gland vs. isolated ductal epithelium (Fig. 9 and 10) and TEB regions vs. mid-gland regions (Fig. 8). These results are encouraging for the next task of the research that will focus on identification and characterization of unique nuclear matrix proteins in TEB and TD cell nuclei involving micro-sequencing and antibody generation.

Task 2a. Cloning and sequencing of unique DD-PCR products.

Of the 32 clones identified by DD-PCR to date, 12 have been amplified from polyacrylamide gel fragments (e.g. see Fig. 6B) and subcloned into the pCRTRAP vector (GenHunter). Four of these clones, EDD-C2, EDD-C13, EDD-C16 and EDD-17 have been completely sequenced, while sequencing continues on the remaining eight clones. Two of the sequenced clones, EDD-C17 and EDD-C2, were highly homologous to sequences in the Genbank data base. EDD-C17 appears to be cytochrome C oxidase, a mitochondrial enzyme involved in energy metabolism. EDD-C2 has an 84% identity to the human p190-B cDNA which has been recently cloned by Y. Yamada (NIDR) [Fig. 6C]. We hope to obtain specific antisera from Dr. Yamada to help determine the localization of p190-B expression in the mammary gland. p190-B is a new member of the RhoGAP family. Members of this family are GTPase Activating Proteins (GAP) which aid in the catalyzation of GTP to GDP specifically by Rho. Rho is a GTPase which regulates actin stress fiber formation. Besides serving as a GTPase activating protein, p190-A, a homologous protein, is thought to interact with p120, a rasGAP. In this way, p190 may bridge the intracellular signalling pathways between ras and rho. Additionally, the p190 sequence contains a region which is identical to GRF-1, a transcriptional repressor of the glucocorticoid receptor. Thus, p190 may also serve to link membrane signalling to nuclear events.

Construction of a rat postnatal day 45 virgin mammary gland cDNA library has also begun, to permit the isolation of full length cDNA clones identified by DD-PCR. The fourth mammary glands were dissected from nulliparous 45 day old Wistar-Furth rats and total RNA was isolated. mRNA was isolated using the Promega PolyA tract isolation system. The cDNA library was prepared from this mRNA by oligodT priming (Clontech, Great Lengths cDNA Synthesis Kit). High molecular weight cDNAs were selected through size selection by gel filtration. The size and integrity of the cDNA was assessed by gel electrophoresis and EcoRI adapters were ligated to the cDNA prior to integration into the λZAP library system (Stratagene). Currently, the library is being amplified and titered.

CONCLUSIONS

During the first year of this proposal we have validated the feasibility of manually dissecting end bud, mid-gland and stromal subfractions from the mammary gland and the isolation of intact RNA and nuclear matrix proteins from these fractions. We have performed confocal analysis and differential display PCR and 2D-PAGE analyses and identified differences in gene expression in these fractions. In the following year, the specificity of the TEB cDNA clones will be verified by northern analysis using TEB, mid gland and stroma RNA fractions. If mRNA transcripts can not be detected by northern hybridization, RNAse protection assays or RT-PCR may be employed. Characterization of TEB specific clones will continue by studying the developmental fate of these markers by use of developmental northerns with RNA samples representing young virgin, old virgin, pregnant, lactating, involuting and hormone-treated animals. Tissue distribution of clone expression will be determined by northern blots of RNA from multiple rat tissue samples. In particular, we will focus our attention on studying the temporal and spatial pattern of expression of the p190-B clone. Additionally, clones without homology to Genbank sequences will be used to probe the 45 day virgin mammary gland library to isolate full length cDNAs. Work will also begin on *in situ* hybridization experiments to explore clone expression patterns in 45 day rat mammary glands. Transcripts will be localized on frozen mammary gland sections using biotinylated- or digoxygenin-labeled probes. Finally, the reproducibility of the differences in nuclear matrix proteins in the different fractions will be confirmed and candidate proteins isolated and microsequenced.

REFERENCES

None.

APPENDIX

Figures 1-10 attached.

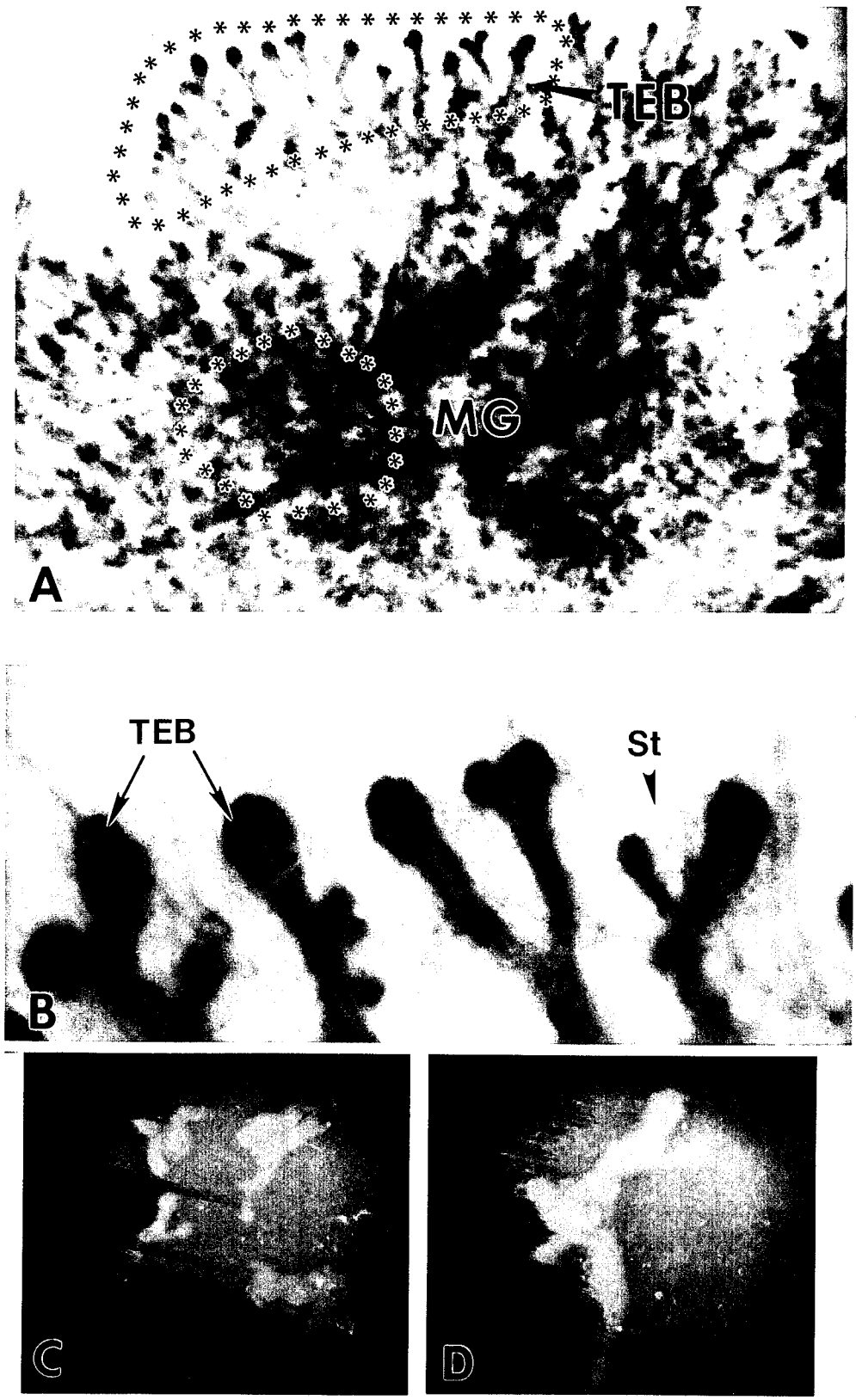


Figure 1. **A**. Survey of mammary gland from 50 day old rat injected with trypan blue. From these types of preparations, it is possible to dissect regions rich in TEBs from those in the mid-gland (MG). **B**. A higher magnification of the TEBs and surrounding stroma (St). **C-D.** After dissection and digestion with 0.5% collagenase, the TEBS can be seen relatively free of stromal cells. This sample was used for 2-D PAGE analysis.

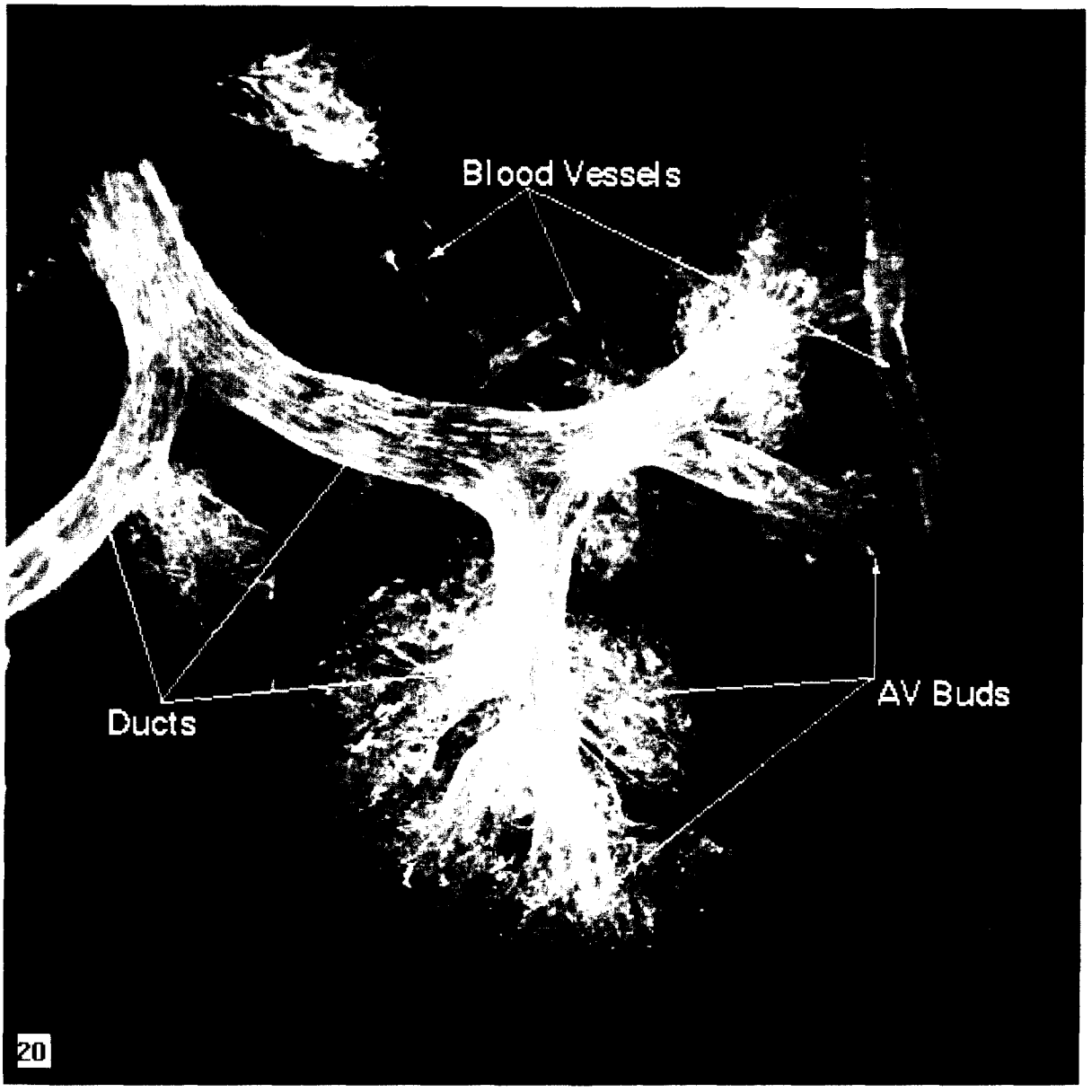


Figure 2. A confocal microscope projection of 89 optical sections (1.8 μ steps) of mammary gland stained for actin. Note details of actin bundles in myoepithelial cells of the duct. Blood vessels, ducts and AV buds are clearly shown.

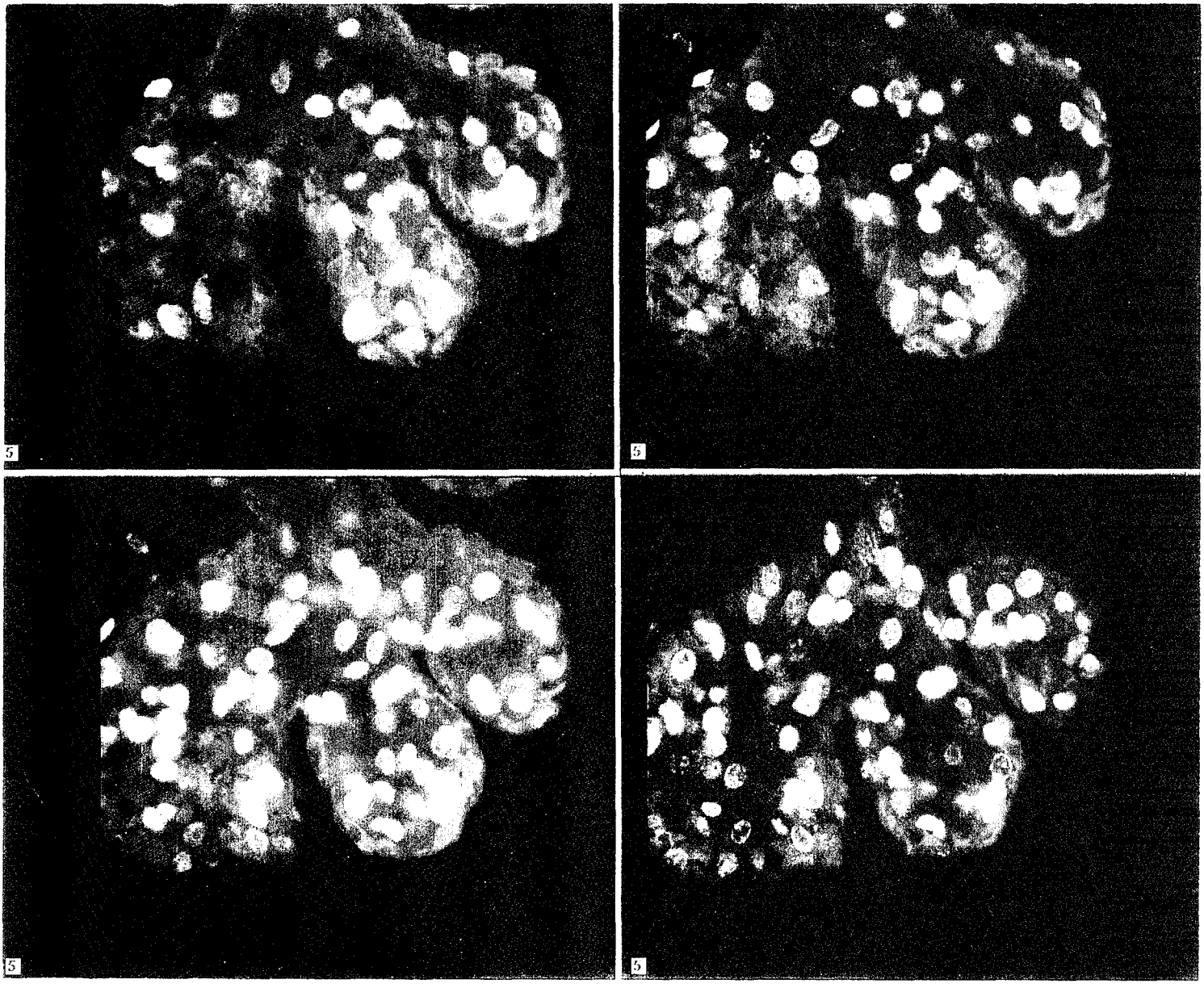


Figure 3. Four optical sections (1.10 μ step) through three TEBS of a 50 day old rat injected with BrdU and fixed 3 hr later. Stained with FITC-anti BrdU and Texas Red anti-keratin and pseudo colored green BrdU and red for keratin. Numerous nuclei show positive staining indicating an elevated labeling index in this region.

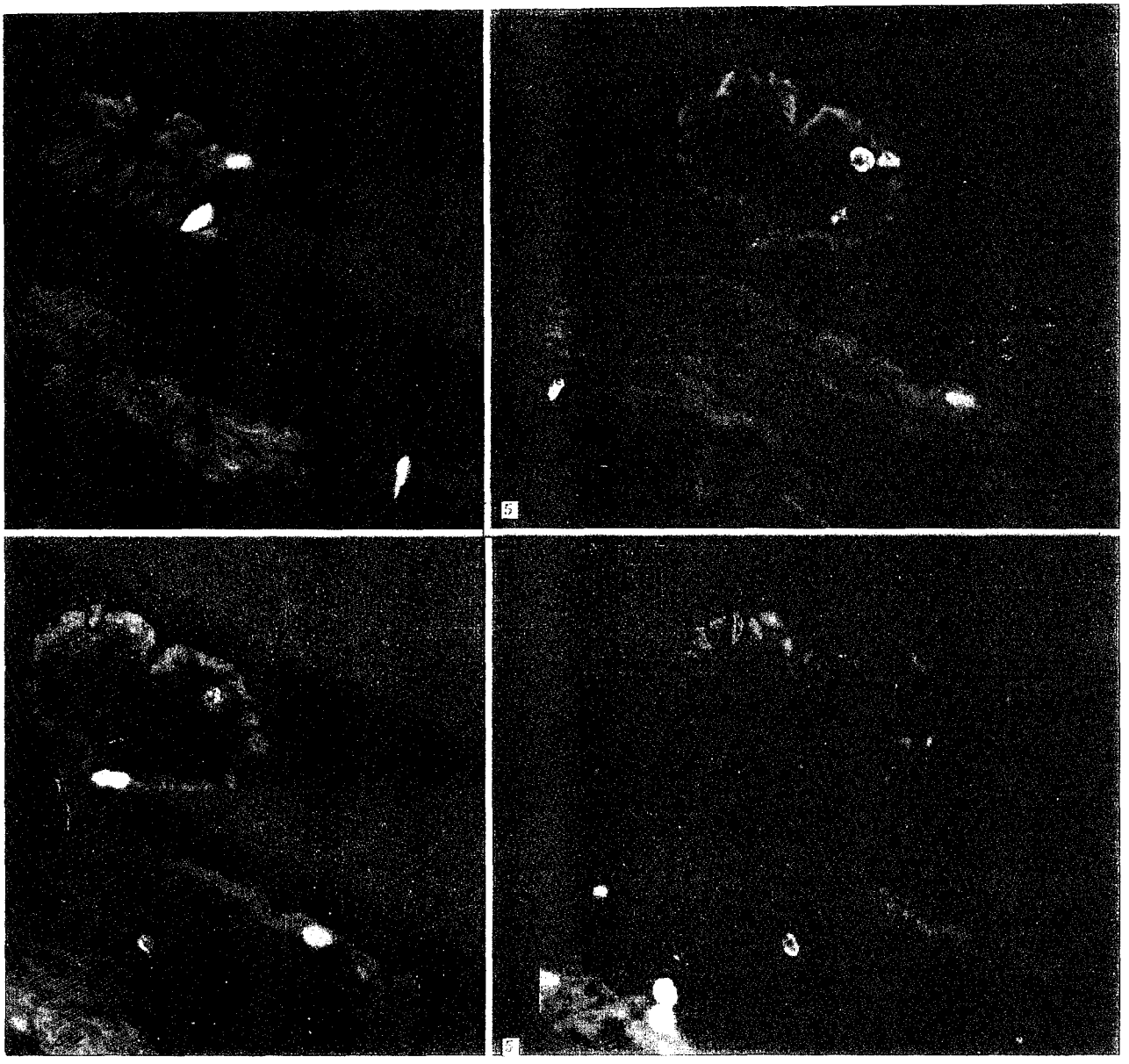


Figure 4. Four optical sections (1.10 μ step) through ducts and alveolar buds of the same preparation as shown in Figure 6. Only a few nuclei are labeled indicating a low LI for this region.

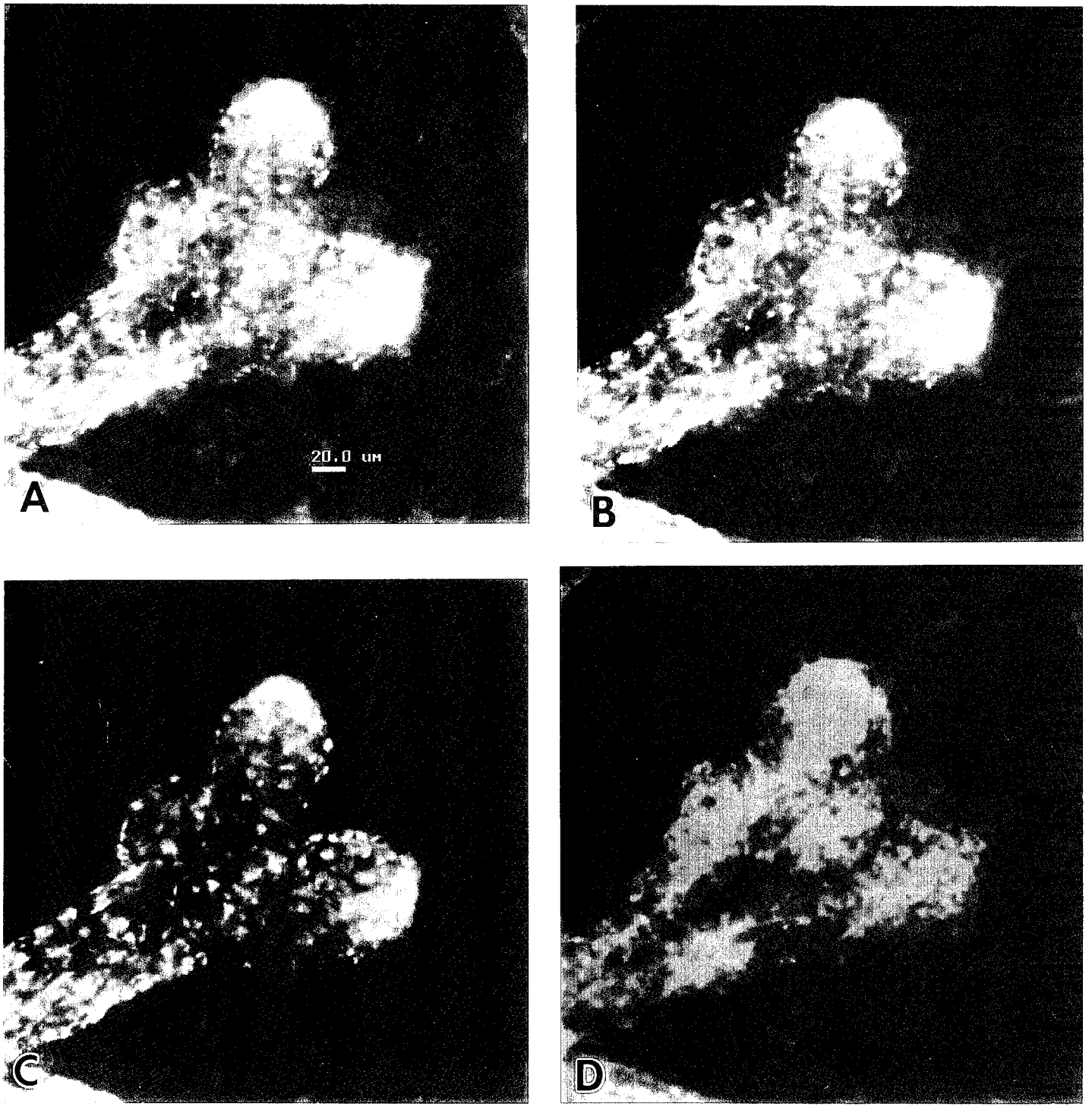
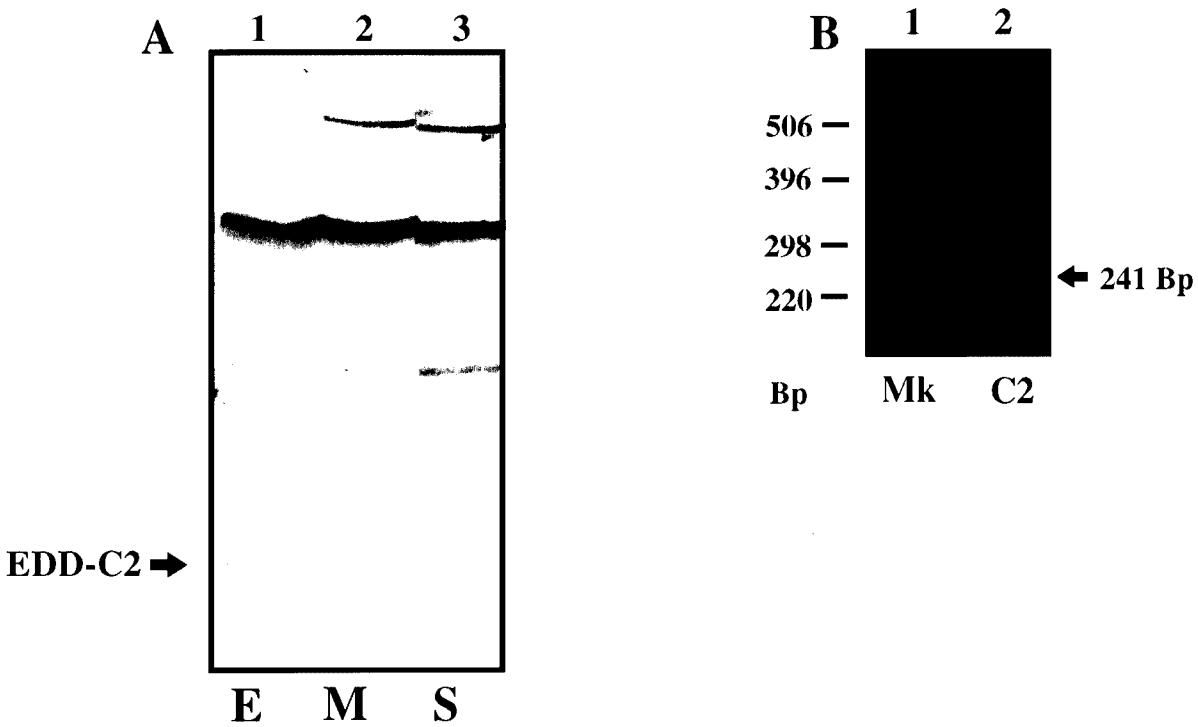


Figure 5. Images of TEB from mouse mammary gland acquired on a DeltaVision deconvolution microscope. This preparation was triple-stained for keratin 14 (green), actin (red) and DNA (blue). A projection showing all three stains is shown in frame A. Frame B shows only two stains (actin and K14), frame C shows K14 alone and frame D, actin alone.



C

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p190-B    AAGCTCTCAGAGGAGTATTGGAATCAG-TTAAACACAATTTGGATGTGGTGAGCCCAATTCCTGC
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EDD-C2    AAGCTTAACGAGGAGTATTAGAATCAGGTTAAACACAATTTAGATGTGGTGAGCCCAGTTCCTAT
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          CAATAAGGACTTAT---CAGAAGCTGAC-TTGAGAATTGTCATGTGCGCCATGTGTGGAGATCCA
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Figure 6. Identification of End Bud Differential Display Clone C2 (EDD-C2). (A) Differential Display of mRNA from the End Bud (E, lane 1), Mid Gland (M, lane 2) and Stroma (S, lane 3) of 45 day virgin rats. The mRNA was reverse-transcribed with the T11C primer and then amplified with the T11C and AP7 primer set. EDD-C2 is indicated by the arrow. (B) 2% agarose gel depicting EDD-C2 reamplification with AP7 and T11C; marker (lane 1), EDD-C2 (lane 2). (C) Sequence alignment of EDD-C2 and human p190-B (RhoGAP).

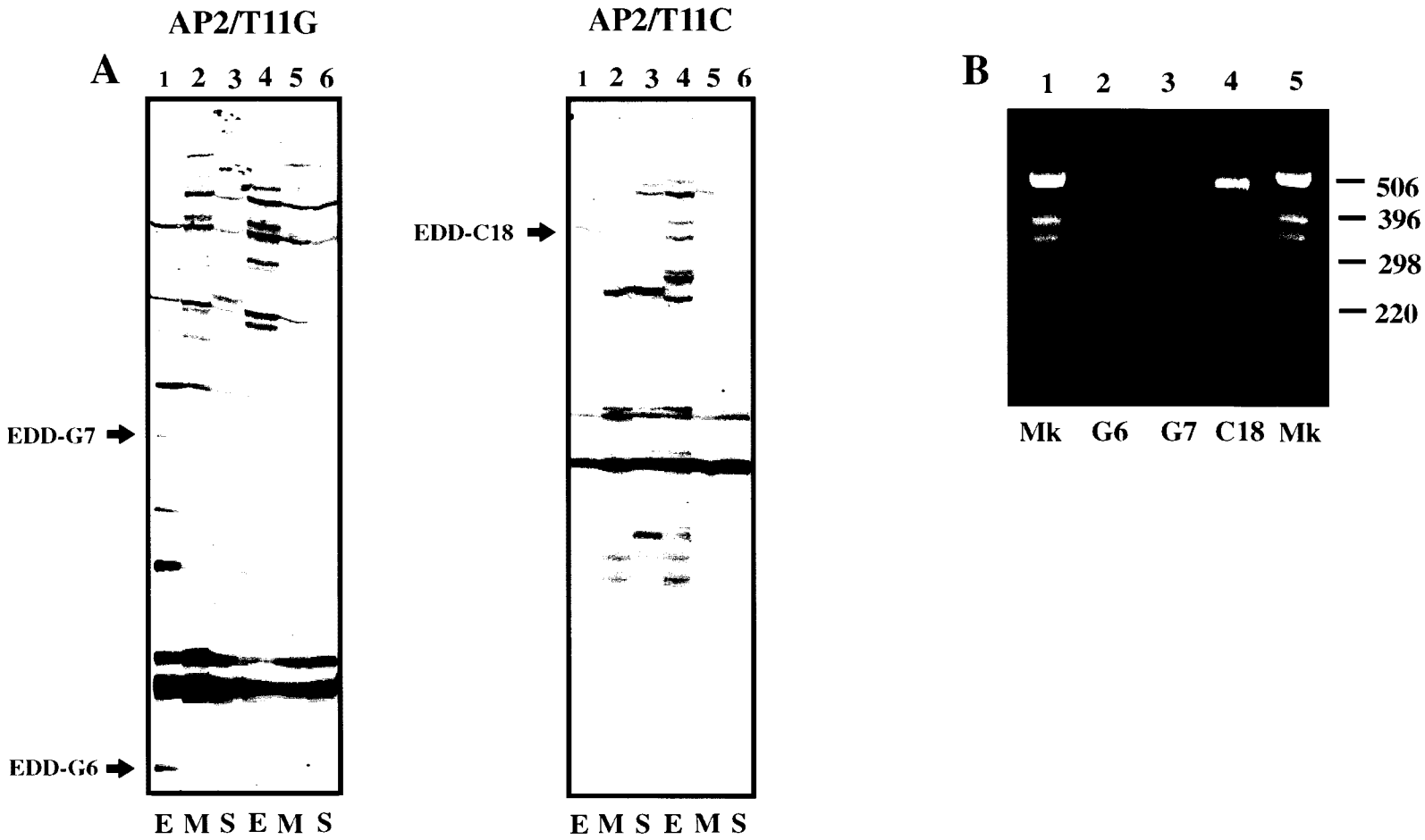

Figure 7. Identification of Differential Display Clones G6 (EDD-G6), G7 (EDD-G7) and C18 (EDD-C18). (A) Differential Display of two separately prepared mRNA sets from the End Bud (E), Mid Gland (M), and Stroma (S) from 45 day virgin rats. The mRNA was reverse-transcribed with the T11G or T11C primer, and then amplified with AP2 and the corresponding 3' primer. Arrows indicate EDD-G6, EDD-G7 and EDD-C18. (B) 1.5% agarose gel depicting reamplification of EDD-G6 (lane 2), EDD-G7 (lane 3) and EDD-C18 (lane 4).

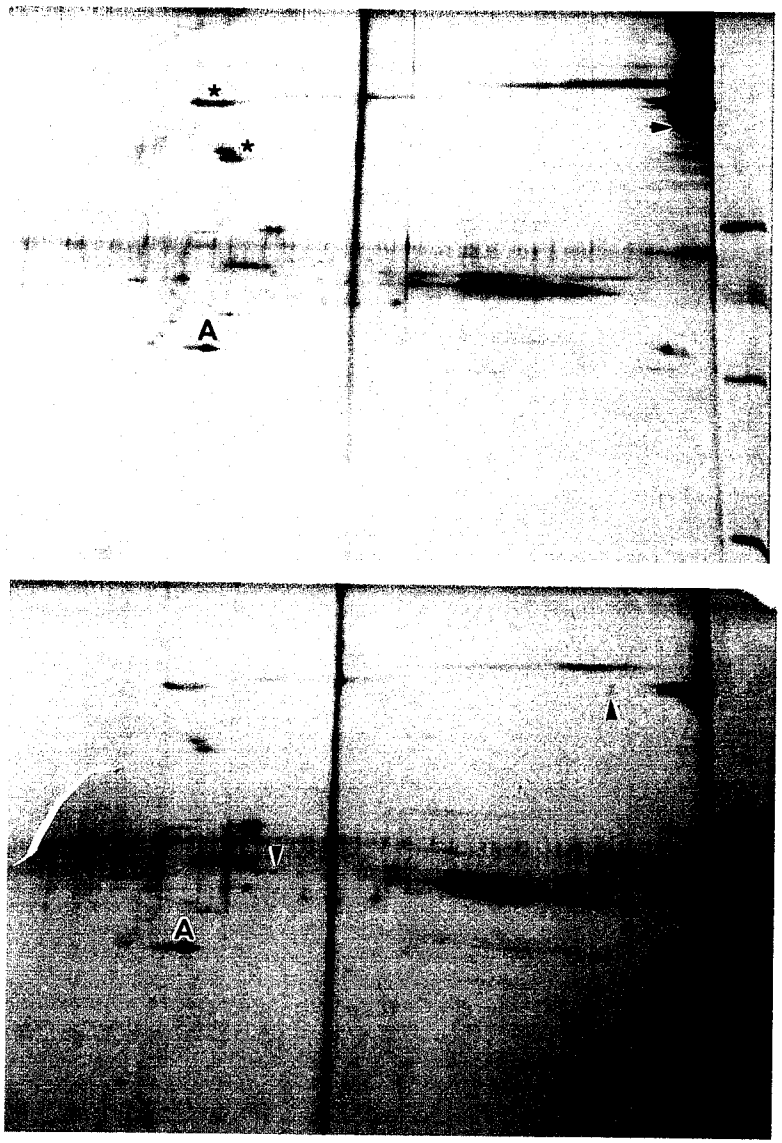

Figure 8. 2-D PAGE of nuclear matrix proteins from microdissected TEB region (top), and the mid-gland region (bottom). The patterns are very similar but at least four proteins are shown that appear to be region specific (arrows). Others varied in amount (stars) when compared to actin(A).

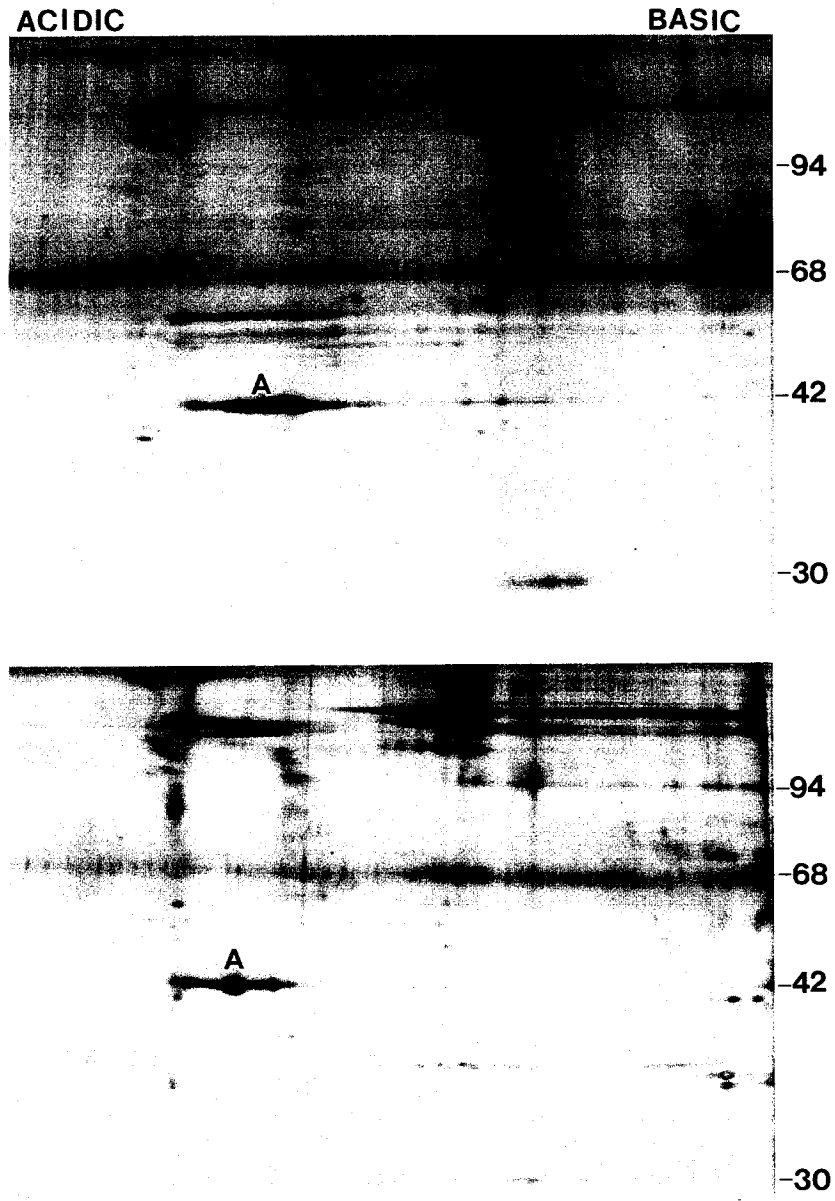

Figure 9. Silver-stained 2-D PAGE of nuclear matrix proteins from isolated rat mammary gland epithelial cells (top) and from whole undissected glands (bottom). As expected, the patterns are similar but differ significantly in some areas. (urea dissolved).

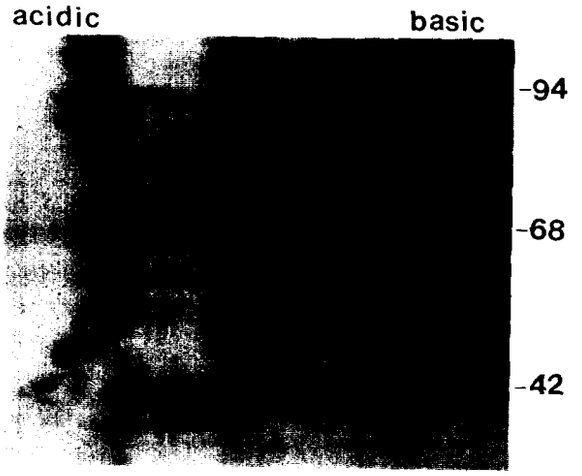

Figure 10. High-resolution, color-based silver staining of 2-D PAGE of nuclear matrix proteins from isolated epithelial cells from 50 day old rat mammary gland (SDS dissolved).